CD4 T HELPER CELL PROFILING IN HEART AND LIVER TRANSPLANT RECIPIENTS: COMPARISON BETWEEN CNI-, RAPAMYCIN- AND ANTI-CD25 mAb-BASED IMMUNOSUPPRESSION

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August 2011

A thesis submitted to McGill University in partial fulfillment of the requirements of the degree of Master in Science in Experimental Surgery.

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ISBN: 978-0-494-84223-2

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ISBN: 978-0-494-84223-2

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Acknowledgements

This research is the product of two years of work at the Surgical Research Laboratories at the Royal Victoria Hospital. I would first like to thank Dr. Jean Tchervenkov and Dr. Marcelo Cantarovich for their continuous support, enthusiasm and motivation.

In addition I would like to thank Shuqing Liu for teaching me all the experimental procedures performed in this project, without her this would not have been possible.

Dr. Minh-Tri Nguyen provided invaluable knowledge and insight regarding data analysis and statistics which I appreciate tremendously.

Catherine Flemming was extremely helpful in consenting patients and collecting blood for the experiments.

Abstract

The use of calcineurin inhibitors (CNI) is the basis of many immunosuppressive regimens because of its clinical success and contribution to the remarkable increase in the rate of allograft survival. However, standard recommended doses of cyclosporine and tacrolimus are associated with nephrotoxicity, resulting in long term renal dysfunction. An ideal strategy would employ a CNI-free regimen. Attempts to convert heart and liver transplant patients to rapamycin resulted in an improvement in renal function however the incidence of side effects lead to dose reduction or drug discontinuation in many cases. Anti-CD25 monoclonal antibodies (mAb) have been used in induction immunosuppression and have been shown to reduce the incidence of acute rejection in solid organ transplantation. We have used anti-CD25 mAb for maintenance immunosuppression as a substitute to CNI in patients with chronic kidney disease. It was of our particular interest to examine the impact of immunosuppressive medications such as CNI, rapamycin and anti-CD25 monoclonal antibody on naive T helper cell differentiation. We hypothesize that as a result of their diverse mechanisms of action, the effect of immunosuppressive medication on CD4 T helper cells (Th1, Th2 and Th17) results in variability. We found that Th1 and Th17 cells as a proportion of CD4 cells and total lymphocytes in heart transplant patients are lower in CNI patients when compared to anti-CD25 mAb patients and healthy controls. We also found that Th17 cells as a proportion of CD4 cells in liver transplant patients are higher in patients on anti-CD25 mAb and rapamycin when compared to healthy controls. We wanted to determine if this variability had implications for graft rejection as well as two common complications of immunosuppression which are infection and malignancy however we did not find any correlation between proportions of T helper cells and rates of rejection, infection and malignancy.

Resumé

L'utilisation des inhibiteurs de la calcineurine (ICN) est la base de plusieurs traitements immunosuppresseurs en raison de son succès clinique et de sa contribution à l'augmentation remarquable du taux de survie des allogreffes. Cependant, les doses standards recommandées de cyclosporine et de tacrolimus sont associées à des néphrotoxicités, résultant en un dysfonctionnement rénal à long terme. Une stratégie idéale serait d'employer un régime sans ICN. Des tentatives de transférer les patients ayant subis des transplantations cardiaques et hépatiques à la rapamycine ont entraîné une amélioration de la fonction rénale. Cependant, l'incidence des effets secondaires a conduit à la réduction de la dose ou l'arrêt du médicament dans beaucoup de cas. Les anticorps monoclonaux anti-CD25 (AcM anti-CD25) ont été utilisés dans l'immunosuppression initiale et se sont dévoilés comme réducteurs du risque de rejet aigu lors de transplantations d'organes solides. Nous avons utilisé AcM anti-CD25 pour des fins d'immunosuppression de maintien comme substitut aux inhibiteurs de la calcineurine chez les patients atteints de maladie rénale chronique. Il était particulièrement dans notre intérêt d'examiner l'impact des médicaments immunosuppresseurs comme les ICN, la rapamycine et AcM anti-CD25 sur la différenciation des cellules T helper CD4. Nous émettons l'hypothèse que, suite à leurs diverses mécanismes d'action, les effets des médicaments immunosuppresseurs sur les lymphocytes T CD4 helper (Th1, Th2 et Th17) sont variables. Nous avons trouvé que les cellules Th1 et Th17 comme une proportion de cellules CD4 et lymphocytes totaux, chez les patients a transplantation cardiaque, sont moins élevées chez les patients ICN comparativement aux patients AcM anti-CD25 et les contrôles sains. Nous avons aussi trouvé que les cellules Th17 comme une proportion de cellules CD4 chez les patients a transplantation hépatique sont plus élevées chez les patients sur AcM anti-CD25 et la rapamycine, par rapport aux contrôles

sains. Nous voulions déterminer si cette variabilité a des implications dans le rejet du greffon ainsi que dans deux complications fréquentes de l'immunosuppression, qui sont les infections et la malignité. Toutefois, nous n'avons pas trouvé de corrélation entre les proportions de cellules T helper et les taux de rejet, d'infection et la malignité.

1. Introduction

Solid organ transplantation has been one of the most innovative therapeutic advances in medicine for the past 60 years. It has progressed to become a routine practice which is clinically effective and life saving and remarkably continues to be a field which is a dynamic work-in-progress. Solid organ transplantation is the process whereby diseased organs are replaced by other organs as a means to restore normal physiologic function. There are different types of transplants based on the origin of the tissue to be transplanted:

- Autograft: transplant of tissue from and to the same person
- Allograft: transplant of an organ or tissue between two genetically non-identical members of the same species. Most human organ and tissue transplants are allografts
- Xenograft: transplant of organs or tissue from one species to another

In the context of allotransplantation, organs that can be transplanted are the heart, kidneys, liver, lungs, pancreas, intestine and thymus. According to the WHO, the kidneys are the most commonly transplanted organs, followed closely by the liver and then the heart[1].
Kidney transplantation is the ideal treatment for end-stage renal disease (ERSD), defined as glomerular filtration rate of <15ml/min/1.73$m^{2.}$ Some common conditions that can lead to ESRD are diabetes mellitus, malignant hypertension, infections, polycystic kidney disease and lupus. Diabetes is the most common cause of kidney transplantation[2]. In 2010, approximately 17,000 kidney transplants were performed in the United States.

Liver transplantation is potentially applicable to any condition that results in irreversible liver dysfunction. This includes cirrhosis and/or hepatocellular carcinoma often attributable to one or more of the following: long term alcohol abuse and long term untreated hepatitis C and/or B infection. In 2010, approximately 6,300 liver transplants were performed in the United States. A heart transplant is performed on patients with end stage heart failure which could be caused by coronary artery disease, cardiomyopathy, heart valve disease with congestive heart failure or congenital heart defects. In 2010, approximately 2,400 heart transplants were performed in the United States[3].

1.1 History of organ transplantation

Throughout time, curiosity has been displayed by humans in the removal of tissue from one site and its transfer to another in the same individual or to others as cosmetic, restorative or therapeutic procedures. Fascinating descriptions exist in mythological, religious or historical literature including archaeological records from Hindu, Greek and Chinese texts dating back to several millennia ago[4]. Such descriptions, although providing evidence of human inquisitiveness and an innovative mindset to improve medical care, bear no literal relationship to the modern sciences that form our current understanding of transplantation. By the premodern era, the early 20th century, successful transplantation of nonvisceral tissues such as human skin and cornea had been reported. In addition, the French surgeon Alexis Carrel had perfected vascular anastomotic suturing methods, vessel reconstruction and cold preservation then successfully performed kidney reimplantation in the neck of the same dog and a few years between dogs. He won the Nobel Laureate Prize in 1912[5]. However despite his technical surgical success, Carrel's observation was that hostile host response to the foreign allograft was

the impeding factor to successful transplantation. Due to the genetic difference between the organ and the recipient, the recipient's immune system will identify the organ as foreign and try to destroy it, causing transplant rejection. In 1954 the first successful kidney transplant by Dr. Joseph Murray and Dr. John Merrill took place in Boston between two identical twins. The graft survived and no immunosuppression was necessary as there were no genetic differences between the recipient and donor. The recipient survived 8 years with no evidence of rejection before succumbing to cardiovascular disease (with intact renal function). Soon after, in the late 1950's, the first successful kidney transplant was performed by Joseph Murray between genetically non-identical twins. The recipient survived 20 years with intact renal function. Joseph Murray won the Nobel Prize in Medicine in 1990. However the need for immunosuppression in subsequent renal transplant patients was apparent. Liver transplantation was developed a few years after kidney transplantation and the first liver transplantation was attempted by Dr. Thomas Starzl at the University of Colorado in 1963. However it resulted in perioperative death of the patient because of overwhelming complications[6]. Unsuccessful attempts were continued in the USA and France between 1963 and 1967. The first one-year survivor of liver transplantation did not occur until 1967[7]. The first heart transplantation was performed by Dr. Christian Barnard in Capetown, South Africa in a cardiomyopathic recipient who survived 18 days. However, an inadequate understanding of the rejection process and the inability to diagnose and treat rejection resulted in a drop from 100 heart transplants in 1968 to just 18 in 1970. It was clear that a more concrete understanding of the complications of transplantation immunology and solutions to the problems of rejection were necessary.

The principle that organ rejection arises because of an immune reaction against the graft was revealed by Peter Medawar first in the early 1940's. This led to the critical idea that in order for the recipient to accept an allograft, their immune system should be weakened. Then, three men, Rupert Billingham, Leslie Brent and Peter Medawar published an article in Nature in 1953 describing how they had isolated the leukocytes from the spleen or bone marrow of adult mice and injected them into the blood of newborn mice. The immune system of the newborn mice was not developed enough to reject the infected cells, and therefore the donor leukocytes engrafted and were thought to have replaced the recipient immune cells. A few years later, this concept was reproduced in adult mice whose otherwise normal immunity had been weakened by total body irradiation, thus leading to the initial concept of immunosuppression applicable to adult humans[8].

1.2 Immunosuppression

An important cornerstone in transplantation was the principle that the recipient's immune system had to be suppressed in order to break the genetic compatibility barrier. In the late 1950's in Boston, sublethal total body irradiation was used to prepare patients for kidney transplantation. 90% of patients died within the month however, the cause of death was a result of the radiation, not allograft failure. It was apparent that cytoablative radiation was too blunt an immunosuppressive instrument and development of pharmacologic immunosuppression was a more practical and safe alternative. Unexpectedly, simultaneous development of antileukemia agents including cyclophosphamide, methotrexate, 6-mercaptopurine and azathioprine was occurring at this time. In animal models, 6-mercaptopurine had proven to delay skin and kidney graft rejection. In 1960, renal transplantation in a human female was managed with

cyclophosphamide and methotrexate. However the patient died after 143 days, despite intermittent rejection managed with prednisone. Subsequent kidney transplantations in the early 1960's where patients were immunosuppressed with either 6-mercaptopurine or its analogue azathioprine, resulted in short term survival which was a concern. The results of these trials did not show potential for these agents in chronic immunosuppression[8]. It was not until 1977 that the face of modern immunosuppression truly changed to reflect what it is today. During this time, the Swiss physician Jean Borel discovered the immunomodulatory properties of cyclosporine. It is a natural peptide product of the fungi Cylindrocarpon lucidum and Trichderma polysporum. The powerful immunosuppressive effects of cyclosporine are directed toward cell-mediated T-helper lymphocyte and lymphocyte-derived antibody synthesis but without the bone marrow suppressive effects of azathioprine or the broad immune non-lymphocyte simultaneous effects of steroids. The use of cyclosporine resulted in tremendous advances in patient survival-trials in the 1980's showed the 1 year graft-survival rate to exceed 89% in kidney transplant recipients and 70% in heart and liver transplant recipients. Significant adverse effects were still common and included nephrotoxicity, neurotoxicity, opportunistic infection, diabetes and B cell lymphoma. These complications were only partially responsive to dose-reduction strategies. In the late 1980's, FK-506 (tacrolimus), which is a product of the fungus Streptomyces tsukubaensis, was clinically investigated in human liver recipients who were experiencing cyclosporine-refractory rejection. Its use resulted in 75% rescue of such allografts[5]. Both cyclosporine and tacrolimus belong to a class of drugs called calcineurin inhibitors (CNI). Cyclosporine is thought to bind to a protein in the cytosol of immunocompetent lymphocytes (in particular T cells) called cyclophilin. This complex of cyclosporine and cyclophilin inhibits the phosphatase calcineurin. Under normal circumstances, calcineurin allows for translocation of the

nuclear factor of activated T cells (NFAT) from the cytoplasm to the nucleus to induce transcription of effector proteins in the immune response, including interleukin-2 (IL-2), a key cytokine. IL-2 is an important target to block for prevention of allograft rejection. T-cell proliferation is triggered by the interaction of IL-2 with its receptor on activated T cells and promotes the growth, differentiation and survival of T cells. Similarly tacrolimus inhibits calcineurin but instead by associating with the protein FKBP1A followed by the binding of this complex to calcineurin, thus preventing IL-2 transcription[9].

Sirolimus, also known as Rapamycin, is produced by a strain of bacteria called Streptomyces Hygroscopicus, isolated from a soil sample. It was originally isolated as an antifungal agent but subsequent studies revealed its antitumor and immunosuppressive activities. Rapamycin is a potent inhibitor of antigen-induced proliferation of T cells, B cells and antibody production. Experiments in animal models of allotransplantation demonstrated its potent immunosuppressive activity and consequent approval for human use. Rapamycin forms an immunosuppressive complex with the protein FKBP12 which is part of the family of FK binding proteins. Tacrolimus exerts it effect by interacting with FKBP1A, which is also part of that family. The rapamycin:FKBP12 complex binds to a pivotal regulator of cell growth and proliferation called mammalian target of rapamycin (mTOR), thereby inhibiting its activity. By interfering with the function of mTOR, rapamycin inhibits the mTOR mediated signal-transduction pathways, resulting in the arrest of cell cycle at the juncture of G1 and S phase, thus inhibiting T-lymphocyte proliferation[10]. IL-2 is an important target to block for prevention of allograft rejection. T-cell proliferation is triggered by the interaction of IL-2 with its receptor on activated T cells[9].

The discovery of mycophenolic acid as an immunosuppressant was essential for the introduction of mycophenolates to the field of transplantation. In particular, the use of mycophenolate mofetil (MMF), a morpholino ester prodrug of MPA signified a great advance in the prevention of allograft rejection. MPA was first isolated from Penicillium spp. and was found to posess immunosuppressive activity when used in the treatment of psoriasis. It's mode of action is to reversibly inhibit the enzyme inosine monophosphate dehydrogenase (IMPDH), a critical enzyme for the de novo synthesis of the purine building blocks of DNA (guianine and adenine). Two major pathways are involved in purine synthesis – the de novo pathway and the salvage pathway. Lymphocytes depend on the de novo pathway, thus the effects of MMF on purine synthesis is significant as they have no alternative way of producing adequate amounts of purines if IMPDH is unavailable. Therefore, MMF prevents proliferation of T and B cells[11a].

Monoclonal antibodies (mAb) have also been used to prevent organ allograft rejection. Antibodies are produced by B lymphocytes in vertebrates and can bind any antigenic determinant (epitope) in the organism. Thus, the remarkable specificity of antibodies made them promising agents for human therapy. In 1975, Cesar Milstein and Georges Kohler fused short-lived, highly specific lymphocytes with the cells of a myeloma (cancer cells that can reproduce indefinitely). These hybridomas secreted antibody to a single antigen, and perpetuated themselves due to the immortal characteristics of the myeloma. This enabled production of large quantities of pure antibody against single antigen characteristics (monoclonal antibodies)[11]. For this work, Milstein and Kohler received the Nobel Prize in 1984. Essentially, monoclonal antibodies, in the context of transplantation, can be used as antigen-specific immunosuppressants to block specific cell receptors and thus prevent the action of certain interleukins that activate T cells. The initially approved monoclonal antibody OKT3 targeted the CD3 element of the T-cell

antigen receptor complex. Anti-CD25 mAb are used to selectively block IL-2 receptors (CD25 is the receptor for IL-2) thus preventing the IL-2 mediated stimulation of lymphocytes[12]. Daclizumab is an anti-CD25 mAb and exerts its immunosuppressive effects through competitive antagonism of the alpha subunit of the high affinity IL-2 receptor. This subunit is a valuable target for immunotherapy as it is not expressed on many normal, resting T-cells however is expressed on abnormal T cells participating in allograft rejection[13].

1.3 The immune system and T helper cells

In order to fully understand allograft rejection and elucidate the mechanisms behind immunosuppressive drug mechanisms of action and their side effects, it is important to understand how the immune system works and how these drugs work in context. The immune system protects organisms from foreign organisms. The innate immune system provides immediate defence in a non-specific manner and is found in all plants and animals. However it does not confer long-lasting or protective immunity to the host. The major functions of the vertebrate innate immune system include: recruiting cytokines to the site of injury, activating the complement cascade and activation of the adaptive immune system via antigen presentation[14]. The adaptive immune system evolved in early vertebrates and allows for immunological memory, where each pathogen is "remembered" by a signature antigen. This antigen-specificity requires the recognition of specific "non-self" antigens during antigen presentation which generates responses tailored to specific pathogens. The ability to mount these tailored responses is maintained in the body by "memory cells". Should a pathogen infect the body more than once, these specific memory cells are used to eliminate it. The cells of the adaptive immune system are called lymphocytes[15]. B cells and T cells are the major types of lymphocytes. B cells are involved in making antibodies via the humoral immune response. The immune response is

mediated by these antibodies. T cells however are involved in cell-mediated immune response which is not mediated by antibodies but by the activation of immune cells. A subset of T cells thought to be involved in the immune responses seen in transplantation and are the target of many immunosuppressive drugs are called T helper cells, also known as CD4 T cells because of the CD4 surface marker. Unlike many cells involved in cell-mediated immunity, these cells have no cytotoxic or phagocytic activity, they cannot kill cells directly however are essential in activating and directing other immune cells and play a central role in immune protection. They act via their capacity to help B cells make antibodies, induce macrophages to develop enhanced microbicidal activity and to recruit other leukocytes such as neutrophils, eosinophils and basophils to sites of infection and inflammation. One of their most significant roles is the production of cytokines and chemokines which are immune chemicals that orchestrate many key immune responses. The pioneering work of Mossman and Coffman in 1986 showed that long term CD4 T cell lines could be subdivided into 2 groups, those that made interferon-gamma (IFNγ) as their signature cytokine and those that produced interleukin-4 (IL-4)[16]. However it has since been realized that CD4 T cells are not a unitary set of cells but represent a series of distinct cell populations with different functions. Naïve CD4 T cells can differentiate into a number of cell types including T helper 1 (Th1), T helper 2 (Th2), T helper 17 Th17 and regulatory T cells (Treg). The three T helper cells are considered to be “effector T cells”. Regulatory T cells are a specialized subpopulation of T cells that act to suppress activation of the immune system to maintain homeostasis and tolerance to self antigens. In this way they differ from effector T cells which act to help other immune cells exert their actions. The diverse fates of naïve CD4 T cells are established by the pattern of signals they receive upon interaction with innate immune cells that express antigens, costimulatory molecules and inflammatory cytokines:

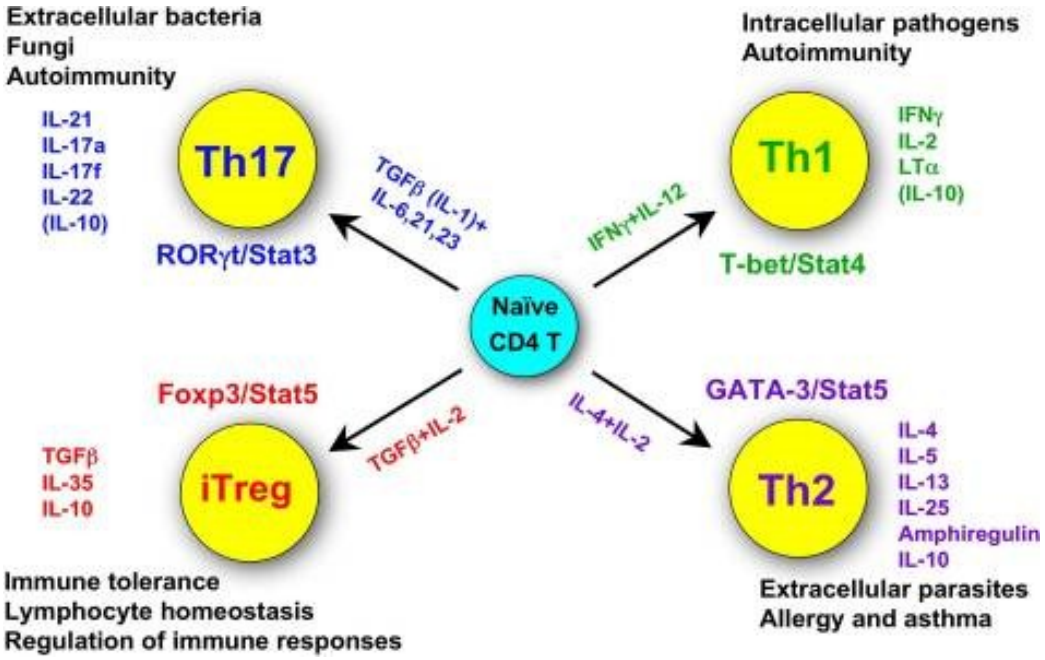


Figure 1.1: Zhu J, Paul WE. CD4 T cells: fates, functions, and faults. Blood 2008(112): 1557-1569

It was originally thought that Th1 and Th2 cells were the only two effector T cells with Th1 cells regulating cellular immunity and Th2 cells regulating humoral immunity. IL-12 regulates Th1 differentiation via activation of the transcription factor STAT4. The transcription factor T-bet is considered to be a key regulator of Th1 differentiation via the potentiation of IFNγ production and suppression of Th2-associated cytokine expression. IL-4 in contrast, drives Th2 differentiation through the actions of STAT6 and GATA3 which is a key regulator of Th2 differentiation via potentiation of IL-4 and suppression of IFNγ. Differentiation of Th1 cells is stimulated by IFNγ and IL-12 and a positive feedback loop is demonstrated as Th1 cells produce predominantly IFNγ as their signature cytokine. Similarly, Th2 cell differentiation is stimulated by IL-4 which is also its signature cytokine[16].

Immunologists and physicians traditionally attributed many autoimmune diseases including multiple sclerosis and rheumatoid arthritis as well as allograft rejection to the action of Th1 cells as IFNγ is considered to be a pro-inflammatory cytokine. However, upon further inspection it was seen that interleukin 17 (IL-17), produced a new Th lineage called Th17 was critical in these pro-inflammatory pathologies previously attributed solely to Th1[17]. In 2006 it was reported that Th17 could be induced in vitro from naïve mouse CD4 T cells by stimulation through their T-cell receptor (TCR) in the presence of IL-6 and TGF-β. RORγT was identified as the master regulator gene for Th17 cells. More work revealed that IL-6 and TGF-β are also critical in human cells for Th17 differentiation. IL-21 produced by Th17 cells induced during the course of their differentiation, fulfills the role of a positive feedback stimulator, showing that Th17 development has logic similar to that of Th1 and Th2 cells. The biological function of IL-17 indicates that IL-17-producing effector T cells are a distinct T helper cell subset[16]. IFN-γ produced by Th1 cells is known for its importance in antigen presentation and cellular responses to intracellular bacteria and viruses. The cytokines derived from Th2 cells have essential functions in humoral immunity and allergic reactions. IL-4 plays a central role in B-cell proliferation and immunoglobulin class-switching and can be both immunosuppressive and immunostimulatory. IL-17 is important in tissue inflammation[17]. It is considered a proinflammatory cytokine with a number of different effector functions as seen in the figure below:

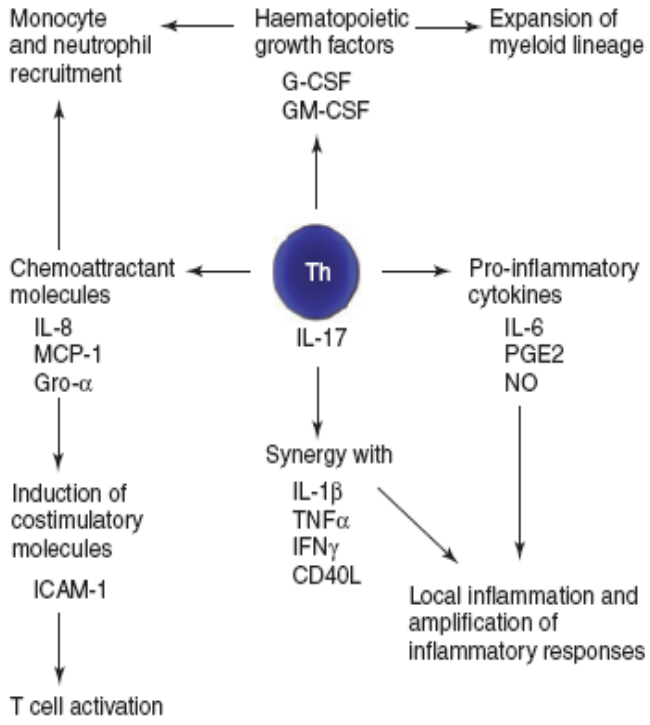


Fig. 1. Proinflammatory effects of interleukin-17.

Figure 1.2: Afzali B et al. The role of T helper 17 (Th17) and regulatory T cells (Treg) in human organ transplantation and autoimmune disease. Clin Exp Immunol. 2007; 148(1): 32–46

Allorecognition pathways are central to rejection however, the effector mechanisms that mediate graft rejection are not clearly defined. In order to improve efficacy and specificity of immunomodulatory therapies it is important to gain a greater understanding of the CD4 effector T-cell subsets that have been implicated in the process of rejection. In addition, immunomodulatory therapies carry a number of risks such as increased incidence of malignancy and opportunistic infection and in order to mitigate these problems, knowledge of effector T-cells is important.

1.4 T helper cells in allograft rejection

As noted, Th1 responses had been held responsible for a wide range of autoimmune and inflammatory diseases which were not attributable to Th2 responses. It has since become apparent that Th17 cells are culpable in a number of these cases. This has in part followed the discovery that IL-12 and IL-23 which promote Th1 and Th17 differentiation respectively are heterodimeric cytokines that share the same p40 subunit. This common subunit can be pared with either p35 to form IL-12 or p19 to form IL-23. Earlier studies in which p40 was deleted or neutralized resulted in clinical improvement in autoimmune disease models which were thought to require Th1 responses, however in subsequent models, knocking out p35 alone did not replicate these results and sometimes even exacerbated disease[17]. In addition, neutralizing or deleting IFNγ was shown to worsen tissue injury in another autoimmune condition which had thought to be Th1 mediated. Recently it has been shown that p19 is essential in the induction of these autoimmune conditions suggesting IL-23-driven Th17 responses are more important than Th1. These observations raised questions about the roles of Th1 and Th17 in allograft rejection. There is a range of clinical data that suggests the presence of Th1 and Th17 cells during allograft rejection, based on the presence of their signature cytokines. IFNγ is found to be lowest in the serum of paediatric liver transplant patients who tolerated their graft without any episodes of rejection[18]. However it does not appear to be a positive indicator of rejection in all cohorts either. In another study it was found that serum IFNγ levels were significantly elevated at 24 months following renal transplantation in patients without rejection episodes when compared to both healthy controls and patients with chronic rejection[19]. IL-17 has been detected by immunofluorescent staining in acutely rejecting human renal transplant biopsies but not in healthy kidneys or pretransplant biopsies[20]. In addition, elevated IL-17 mRNA has been found

in renal biopsy specimens and urinary sediment from patients with borderline rejection when compared to control samples without any evidence of rejection[21]. However it is important to note that direct evidence demonstrating the specific roles of Th1 and Th17 in these studies is lacking because IFNγ and IL-17 are produced by CD4- cells as well such as CD8+ and NK cells[16].

IL-4 is the signature Th2 cytokine and plays a crucial role in B-cell proliferation, immunoglobulin class-switching and the survival and differentiation of T cells. In transplantation, there is evidence that IL-4 can be both immunosuppressive and immunostimulatory. Th2 cytokines are thought to blunt the severity of allograft rejection by inhibiting Th1-mediated cytotoxic T-cell activity. Although tolerising immunosuppressive therapies often downregulate Th1 but not Th2 responses, Th2 cytokines are not necessarily an indicator of graft survival and can occasionally induce rejection[22]. Despite that, there are studies that demonstrate the protective effect of Th2 cytokines on the allograft. Systemic treatment of adult recipients of neonatal cardiac allografts with IL-4 significantly delayed rejection and inhibited Th1 responses within the graft, lymph nodes and spleen[23]. In a rat liver allograft rejection model, donor treatment with IL-4 induced long-term acceptance[24]. However there is also some evidence that in some circumstances, IL-4 can drive Th1 responses during rejection. It has been shown in that Th2 polarized cell lines can promote rejection in immunodeficient mice[25]. In addition, in renal transplant patients, development of chronic rejection was associated with a high IL-4 producing genotype[26].

Both Th1 and Th2 responses can lead to graft dysfunction and destruction however in some cases, the dysfunction resulting from the Th1 response can be ameliorated by IL-4 and vice-

versa[22]. The paradigm for the role of T helper cells in allograft rejection, infection and malignancy is yet to be elucidated.

1.5 Effects of immunosuppression on T helper cells

Considering their prevalent use in the prevention of allograft rejection, it is important to consider the effects of immunosuppressive drugs on T-cells. Two studies show that cyclosporine can inhibit IL-17 production in vitro from CD4 T cells isolated from patients with rheumatoid arthritis[27, 28]. One of these studies also showed a reduction of IFNγ in the presence of cyclosporine[28]. It has also been demonstrated that mRNA of both IL-17 and IFNγ are suppressed in skin biopsies in patients with psoriasis treated with cyclosporine[29]. In a study with a mouse model, cyclosporine and rapamycin were compared and were both found to suppress IL-17 production[30]. It is of interest to study the effects of different immunosuppressive agents on T cells in organ transplant recipients.

1.6 CD4 T helper cell profiling in long term heart and liver transplant recipients on maintenance CNI-, rapamycin- and anti-CD25 mAb based immunosuppression

Renal dysfunction is a well-known side effect of calcineurin inhibitors and can result in end-stage renal disease requiring chronic dialysis in long-term heart and liver transplant patients[31, 32]. It is associated with an increased risk of death[32].

The development of chronic kidney disease (CKD) occurs approximately 3-4 years post heart transplant and maybe present in up to 10% to 40% of patients with very few having normal renal function[32]. Accordingly, reducing the nephrotoxic effects of CNI-based regimens has become a major goal in the treatment of transplant recipients. Reducing CNI doses in most patients has

gained widespread practice however a large study of heart transplant patients found the use of reduced cyclosporine doses had not lead to a reduction in the incidence of end-stage renal disease (ERSD)[33]. The lack of success of this approach emphasizes the need to address a more targeted strategy in dealing with cyclosporine-induced nephrotoxicity. Therefore, an ideal strategy would employ a CNI-free regimen. Previous studies evaluated the conversion from CNI to mycophenolate mofetil (MMF) or Rapamycin in heart and liver transplant patients. Overall these strategies resulted in an improvement in renal function however the incidence of side effects range between 8% and 76% which may lead to dose reduction or drug discontinuation[32]. Anti-CD25 monoclonal antibodies are widely used in induction immunosuppression[34] and have been shown to reduce the incidence of acute rejection in recipients of solid organ transplants. In a prior report of a single-center evaluation of 55 heart transplant recipients, there was a striking decrease in the rate of acute rejection, by a factor of 2.8, among patients receiving daclizumab (a monoclonal antibody against the alpha-subunit of the IL-2 receptor) with standard triple immunosuppressive therapy (cyclosporine, MMF, prednisone) versus control who only received standard triple immunosuppressive therapy. Additionally, the administration of daclizumab was not associated with any detectable signs of the cytokine release syndrome or allergic responses. The incidence of infection or cancer was not higher in the daclizumab group than in the control group[35].

We have used anti-CD25 mAb for maintenance immunosuppression in a novel strategy as a substitute to calcineurin inhibitors (CNIs) in heart and liver patients with CKD. Preliminary data shows that the use of anti-CD25 mAb allowed recovery of renal function in both the initial post-operative period and in a long term context while preventing acute rejection[32, 36].

It is of particular interest to examine the impact of immunosuppressive medications such as CNI, rapamycin and anti-CD25 monoclonal antibody on naive T helper cell differentiation. As a result of their varied mechanisms of action, the effect of the immunosuppressants on CD4 T helper cells could vary, thereby affecting the frequency and severity of graft rejection as mentioned previously. In addition, two common complications of the immunosuppression used to maintain allograft function are infection and malignancy. This is as result of impairment of the inflammatory responses that would otherwise be intact to respond to such pathologies. Therefore it is also of interest to observe if varied proportions of CD4 T helper cells influence rates of infection and malignancy.

Purpose of the study: To assess the difference in Th1, Th2 and Th17 cell populations in heart and liver transplant patients on maintenance CNI-, rapamycin- and anti-CD25 mAb-based immunosuppression.

We also reported the incidence of rejection, infection and malignancy for the patients in each of the three groups of immunosuppression protocols. However because of the small sample size, the effect could not be statistically assessed as an absolute or direct correlation with CD4 T cell subpopulations.

2. Materials and Methods

2.1 Subjects

Twenty-eight long term heart transplant patients, fifteen long term liver transplant patients and nine non-transplanted controls were enrolled. Peripheral blood was drawn and immediately collected in green capped, heparinized tubes from all subjects at the outpatient clinic after obtaining consent. Four groups were compared for heart and liver transplant patients. The first group (n=11 for heart, n=5 for liver) were patients converted from CNIs to humanized anti-CD25 monoclonal antibody (daclizumab) for greater than six months due to chronic kidney disease. The second group (n=12 for heart and n=4 for liver) were on CNIs. The third group (n=5 for heart and n=5 for liver) were also patients converted from CNIs to Rapamycin. The fourth group (n=6 for heart, n=3 for liver) were non-transplanted, healthy controls. Heart transplant patients in the daclizumab group were initially on CNI for a mean of 13.5 years (range of 8-22 years). They were subsequently switched to maintenance daclizumab for a mean of 2 years (range of 1-6 years). Patients in the CNI group have been on CNI since their transplant for a mean of 9 years (range of 2-13 years). Liver transplant patients in the daclizumab group were initially on CNI for a mean of 9.4 years (range of 5-16 years).They were subsequently switched to maintenance daclizumab for a mean of 5 years (range of 1-10 years). Patients in the CNI group have been on CNI since their transplant for a mean of 11 years (range 2-19 years). The protocol to obtain human blood samples was approved by the McGill University Health Center Research Ethics Board.

Table 1: Groups of heart transplant patients

	Anti-CD25 mAb	CNIs	Rapamycin	Healthy control
n	11	12	5	6
Age at enrolment (mean)	71	63	72	N/A
M:F	8:3	8:4	4:1	N/A
Post-Tx (range) (mean)	9-25 years 16 years	3-13 years 10 years	10-24 years 17 years	N/A
IS	CNIs → anti-CD25 mAb (daclizumab) for >6 months due to CKD + Rapa (n=3), mycophenolate sodium (n=2), MMF (n=6)	CsA (n=7) or FK (n=5) +MMF (n=11), mycophenolate sodium (n=1)	+ MMF (n=5)	N/A

Table 2: Groups of liver transplant patients

	Anti-CD25 mAb	CNIs	Rapamycin	Healthy control
n	5	4	5	3
Age at enrolment (mean)	82	60	59	N/A
M:F	5:0	3:1	4:1	N/A
Post-Tx (range) (mean)	9-17 years 15 years	3-20 years 12 years	4-25 years 14 years	N/A
IS	CNIs → anti-CD25 mAb (daclizumab) due to CKD for >6 months + MMF (n=4)	CsA (n=2) or FK (n=2) +MMF (n=3), mycophenolate sodium (n=1)	+ MMF (n=4)	N/A

2.2 Human peripheral blood mononuclear cell (PBMC) isolation

PBMCs from patients and controls were prepared by density gradient centrifugation on Ficoll-Paque™ PLUS (GE Healthcare, Uppsala, Sweden). 15 ml of whole blood was mixed with 15 ml of balanced salt solution (ideally 1:1 ratio), in this case phosphate buffered saline (PBS) pH 7.2 (Wisent, St-Bruno, QC) was used. 12 ml of the higher density Ficoll-Paque was layered underneath the solution. Density gradient centrifugation was performed for 40 minutes at 400 RCF (relative centrifugal force) at 21ºC. PBMCs were harvested from the interphase layer after plasma was suctioned out, transferred to a new tube, washed with PBS and spun once at 300 RCF for 7 minutes at 4ºC.

2.3 PBMC stimulation

Cells were then cultured in the presence of culture medium composed of X-VIVO 15 Media (Lonza, Allendale, NJ) and 10% human AB Serum (Sigma, Oakville ON), the activators PMA (50ng/mL) (Sigma, Oakville, ON) and ionomycin (1ug/mL) (Sigma, Oakville, ON) for 5 hours at 37ºC in the sterile incubator. The frozen PMA stock solution was 5µg/µL and the working solution was 50 ng/µL, therefore 1µL of stock solution was added to 100µL of culture medium to create the working solution. Similarly, the frozen ionomycin stock solution was 10µg/µL and the working solution was 1µg/µL which was made by adding 1µL of stock solution to 10µL of culture medium. To inhibit cytokine secretion, monensin (GolgiStop) was added at the beginning of culture. After 5 hours, cells were harvested and washed twice with PBS, suspended in PBS and counted.

2.4 PBMC staining

Cells were then distributed to 5ml polystyrene round-bottom tubes (Becton Dickinson, Franklin Lakes, NJ) for immunolabeling (100uL of approximately 1 million cells per tube). As some antibodies which recognize cell surface markers may not bind to fixed antigen, immunostaining for the surface marker CD4 was performed with FITC conjugated anti-human monoclonal CD4 antibody (eBioscience, San Diego, CA) in unfixed cells prior to staining for intracellular cytokines. The cells were incubated for 30 minutes then fixed and permeabilized with fixation/permeabilization solution (eBioscience, San Diego, CA) for 12 hours. Subsequently, they were intracellularly stained with PE conjugated IFNγ, IL-4 and IL-17A anti-human monoclonal antibodies (eBioscience, San Diego, CA) for 30 min at 4°C for phenotypic determination of Th1, Th2 and Th17 cells. After suspending in 500uL PBS, flow cytometry analysis was performed.

2.5 Flow cytometry acquisition and analysis

Samples were analyzed in a FACSCaliber™ flow cytometer (Beckton Dickinson Immunocytometry Systems, Palo Alto, CA) using Cellquest software. Typically 100,000 events in a "live-gate" mode were acquired. The frequency of cytokine-producing cells was expressed as a percentage of the labelled cells. Samples were analyzed using FlowJo software (TreeStar, USA)**.**

2.6 Statistical analysis

Intergroup comparisons were made with Kruskal-Wallis non-parametric test. Wilcoxon-Mann-Whitney test was used if the Kruskal-Wallis test revealed significant differences at the $p \leq 0.05$ level (SPSS 17 software, Softonic).

3. Results

Using flow cytometry, the isolated mononuclear cells from the peripheral blood sample are sorted one cell at a time based on the specific light scattering and fluorescent characteristics of each cell. Th1, Th2 and Th17 cells were identified based on staining with anti-human CD4 antibody and IFNγ, IL-4, and IL-17 respectively. Th1 cells are characterized as CD4+IFNγ+, Th2 cells are characterized as CD4+IL4+ and Th17 cells are characterized as CD4+IL17+. Results are displayed as a modified scatter plot where one dot is equivalent to one patient. The four groups of patients on the different immunosuppressants are displayed on the x axis and the percentage of T helper cells on the y axis (as a proportion of CD4 T helper cells and total lymphocytes). Proportions of cells were used for assessment as opposed to absolute numbers of cells because lymphocyte numbers varied between patients therefore were not consistent to draw comparable conclusions.

3.1 Th1 cells were lower as a proportion of CD4 T cells in calcineurin inhibitor patients compared to both healthy controls and patients on anti-CD25 mAb. Th1 cells were also lower as a proportion of total lymphocytes in calcineurin inhibitor patients compared to healthy controls

Th1 as a proportion of CD4 T helper cells (Figure 5.1A) was significantly lower in the heart transplant patients on maintenance CNI with a mean of 5.4% when compared to both patients on maintenance anti-CD25 mAb and healthy controls, at 26% and 18% respectively. Additionally, Th1 cells as a proportion of total lymphocytes (Figure 5.1B) were also significantly lower in the heart transplant patients on CNI with a mean of 1.7% when compared to healthy controls at 8.3%. There were no differences seen between patients on Rapamycin and any other group. These results suggest that CNI inhibit the Th1 CD4+ inflammatory responses to a greater extent than anti-CD25 mAb in this population of heart transplant patients. IFNγ plays an important role in antigen presentation and cellular responses to intracellular bacteria and viruses. It is possible that there is an increased potential for infection and malignancy in this population of CNI patients or an increased potential for rejection in the anti-CD25 mAb patients.

3.2 There was no difference observed between groups for Th2 cells as a proportion of CD4 cells and total lymphocytes in the heart transplant patients on CNI, anti-CD25 mAb and rapamycin

No significant differences were observed between groups for Th2 cells as a proportion of CD4 T helper cells (Figure 5.2A) or total lymphocytes (Figure 5.2B). Although the mean percentage of Th2 cells as a proportion of both CD4 cells and total lymphocytes for healthy controls appears to be higher when compared to all groups of immunosuppressed patients, significance is not reached. IL-4 plays an important role in B-cell proliferation and immunoglobulin class-

switching. It is possible that as a result of the lack of variation between Th2 proportions between the groups in this population of heart transplant patients, IL-4 induced B-cell effects do not differ considerably.

3.3 Th17 cells were lower as a proportion of CD4 T cells in heart transplant patients on calcineurin inhibitors compared to both healthy controls and patients on anti-CD25 mAb. Th17 cells were also lower as a proportion of total lymphocytes in calcineurin inhibitor patients compared to healthy controls

Just as with Th1 cells, it was seen that Th17 as a proportion of CD4 T helper cells (Figure 5.3A) was significantly lower in the heart transplant patients on maintenance CNI with a mean of 0.44% when compared to both patients on maintenance anti-CD25 mAb and healthy controls, at 1.61% and 1.13% respectively. Additionally, Th17 cells as a proportion of total lymphocytes (Figure 5.3B) were also significantly lower in the heart transplant patients on CNI with a mean of 0.12% when compared to healthy controls at 0.47%. There were no differences seen between patients on Rapamycin and any other group. Similar to the results seen with Th1 cells, these results also suggest that CNI inhibit inflammatory responses to a greater extent than anti-CD25 mAb in this population of heart transplant patients. IL-17 has many pro-inflammatory effects and has been shown to be elevated in grafts undergoing rejection. Therefore, it is possible that there is an increased potential for infection and malignancy in this population of CNI patients or an increased potential for rejection in the anti-CD25 mAb patients.

3.4 There was no difference observed between groups for Th1 cells as a proportion of CD4 cells and total lymphocytes in the liver transplant patients on CNI, anti-CD25 mAb and rapamycin

No significant differences were observed between groups for Th1 cells as a proportion of CD4 T helper cells (Figure 5.4A) or total lymphocytes (Figure 5.4B). Contrary to the results observed with the heart transplant patients, proportions of Th1 cells were not lower in the CNI patient group. However the liver transplant patients are on lower CNI doses than the heart transplant patients which may be of significance for this observation. As IFNγ is a pro-inflammatory cytokine, it is possible that the potential for infection, rejection and malignancy will remain similar between the groups in this population of liver transplant patients.

3.5 There was no difference observed between groups for Th2 cells as a proportion of CD4 cells and total lymphocytes in the liver transplant patients on CNI, anti-CD25 mAb and rapamycin

No significant differences were observed between groups for Th2 cells as a proportion of CD4 T (Figure 5.5A) helper cells or total lymphocytes (Figure 5.5B). Similar to the results observed with the heart transplant patients, proportions of Th2 cells were the same between groups. Thus it is also possible that among this population of liver transplant patients on different immunosuppressants, IL-4 induced B-cell effects do not differ considerably.

3.6 Th17 cells were higher as a proportion of CD4 T cells in liver transplant patients on rapamycin and anti-CD25 mAb compared to healthy controls. There were no differences observed between groups for Th17 cells as a proportion of total lymphocytes

Th17 as a proportion of CD4 T helper cells (Figure 5.6A) was significantly higher in the liver transplant patients on maintenance rapamycin and anti-CD25 mAb with a mean of 1.59% and 1.52% respectively when compared to healthy controls at 0.62%. No significant differences were observed between groups for Th17 cells as a proportion of total lymphocytes (Figure 5.6B).

Although the trend is toward what is seen for Th17 cells as a proportion of CD4 T helper cells, significance is not reached. These results suggest that rapamycin and anti-CD25 mAb in this population of liver transplant patients promote IL-17 induced inflammatory responses to a greater extent than what is seen in healthy controls. This effect is not observed with CNI patients, possibly as a result of the lower CNI doses. It is possible that there is increased potential for rejection in this population of anti-CD25 mAb and rapamycin patients compared to CNI patients.

3.7 There is no apparent correlation between proportion of T helper cells and observed infection, rejection and malignancy in the heart transplant patients

Despite having a lower proportion of pro-inflammatory Th1 and Th17 cells as a proportion of CD4 T helper cells, the heart transplant patients on CNI did not have higher observed infection and malignancy or lower observed rejection than the patients on anti-CD25 mAb (Figure 5.7). Similarly, despite having a higher proportion of Th1 and Th17 as a proportion of CD4 T helper cells, the patients on anti-CD25 mAb did not have higher observed rejection or lower observed infection and malignancy than the patients on CNI. The patients on rapamycin did not have any significant differences in T helper cell proportions when compared to any other groups and did not appear to have higher or lower observed infection, rejection or malignancy.

3.8 There is no apparent correlation between proportion of T helper cells and observed infection, rejection and malignancy in the liver transplant patients

The liver transplant patients on anti-CD25 mAb and rapamycin had higher Th17 as a proportion of CD4 T cells compared to healthy controls but the patients on CNI did not demonstrate any significant differences in Th17 proportions compared to any other group. The patients on anti-

CD25 mAb and rapamycin did not however have higher observed rejection or lower observed infection or malignancy compared to patients on CNI (Figure 5.8).

4. Discussion

Solid organ transplantation is a therapeutic option for many human diseases. However immunosuppression after solid organ transplantation can be complex. In the past 60 years, there have been significant advances in immunosuppressive therapy and thus the care of patients receiving allografts. Better therapeutic strategies have been associated with improved patient and graft survival rates. However the unfavourable side effects associated with these agents and the risks of long-term immunosuppression present a number of challenges. In particular, CNI are associated with nephrotoxicity and because they are so commonly used, different immunosuppressive strategies must be considered as an alternative.

In allograft recipients, rejection is a familiar concern and additionally, two common post-transplant complications that can arise as a result of immunosuppression are malignancy and infection. Many immunosuppressants depress primarily cell mediated immunity, however, blunted antibody responses and leucopenia may also be a result of the use of these drugs. The depressed immunity can lead to increased susceptibility to bacterial, viral and fungal infections[37]. Immunosuppressed allograft recipients have a 3- to 4-fold increased risk of developing tumours, but the risk of developing certain cancers is increased several hundredfold. Many of the common malignancies, with the exception of skin and lip cancers, seen in the general population are not increased in incidence but there is a higher frequency of some relatively rare tumours[38].

Allorecognition pathways are central to rejection however, the effector mechanisms that mediate graft rejection are not clearly defined. In order to improve efficacy and specificity of immunomodulatory therapies it is important to gain a greater understanding of the CD4 effector

T-cell subsets that have been implicated in the process of rejection. In addition, to mitigate the problems of malignancy and infection, knowledge of effector T-cells is important, especially in the context of immunosuppression. IFNγ and IL-17 have been observed in grafts undergoing rejection and IL-4 is thought to blunt the severity of allograft rejection by inhibiting Th1-mediated cytotoxic T-cell activity. Therefore it was of our particular interest to examine the impact of immunosuppressive medications such as CNI, rapamycin and anti-CD25 monoclonal antibody on naive T helper cell differentiation in heart and liver transplant patients. It was also of interest to observe if varied proportions of CD4 T helper cells influence rates of infection and malignancy. We hypothesized that increased proportions of the pro-inflammatory cytokines IFNγ and IL-17 would result in increased rejection and decreased proportions in increased infection and/or malignancy. We similarly hypothesized that increased proportions of IL-4 would blunt the severity of rejection.

Flow cytometry analysis was used to determine Th1, Th2 and Th17 as a proportion of either CD4 cells or total lymphocytes. The rationale for measuring the cells as a proportion of CD4 cells or total lymphocytes as opposed to absolute numbers of cells was that lymphocyte numbers varied between patients therefore were not consistent to draw comparable conclusions.

The results of this study demonstrate that different immunosuppressants can have varied effects on T helper cells in long term heart and liver transplant patients. Contrary to our expectations however, this does not necessarily mean there is an impact on infection, malignancy and rejection in these patients. The small number of patients studied did not allow us to draw an absolute conclusion however. Risk of rejection, infection and malignancy is multifactorial. Additionally, we did not measure the effect of the different immunosuppressive protocols on

other T cells such as CD8+ or CD4+ Tregs. From our study we observed that the heart transplant patients on CNI did not have a higher incidence of infection and malignancy despite having lower proportions of Th1 and Th17. Similarly the heart transplant patients on anti-CD25 mAb and the liver transplant patients on rapamycin and anti CD-25 mAb did not have a higher incidence of rejection. Anti-CD25 mAbs target the IL-2 receptor alpha subunit. This is a particularly valuable target for immunotherapy because very few normal, resting cells express IL-2Rα. Instead it is expressed largely by abnormal, pathological T cells such as those participating in allograft rejection and autoimmune disease[13]. Therefore, despite having higher proportions of pro-inflammatory effector T cells both heart and liver transplant patients on daclizumab are not rejecting their grafts because it is likely that the T cells present are those with normal function, thereby, also permitting patients to maintain the ability to combat infection and malignancy.

A possible explanation for the absence of rejection is that other endogenous factors are protecting against rejection that could otherwise be exacerbated by pro-inflammatory cytokines produced by Th1 and Th17. Regulatory T cells, which are also CD4+ but are not effector T cells, are associated with the promotion of allograft tolerance. However, regulatory activity is not exclusive to CD4+ T cells as CD8+, CD8+CD28-, TCR+CD4-CD8- (double negative) cells as well as NKT cells have also been shown to have regulatory activity after transplantation[39]. It is likely that regulatory mechanisms of both the innate and adaptive immune systems will contribute to the overall outcome after transplantation.

Another possible explanation is that the cytokines produced by Th1 and Th17 are not solely responsible for provoking rejection. Endothelial cell changes associated with allograft rejection are associated with factors such as P-selectin and ICAM-1 as well as an upregulation of

proinflammatory cytokines such as IL-1(beta) and TNF(alpha)[40] any of which could be associated with promoting rejection.

Finally, measuring the circulating, peripheral blood lymphocytes may not be the most accurate indicator of rejection in a graft. Often, intragraft levels of IFNγ and T-bet are used as indicators of rejection as opposed to serum levels. In a study, urinary protein levels of the IFNγ-induced chemokine MIG (monokine induced by IFNγ) were significantly elevated during episodes of biopsy-proven acute rejection in renal transplant patients[41]. Similarly, T-bet, the Th1-specifying transcription factor, was found to be elevated in renal transplant biopsies during episodes of acute rejection[42]. Elevated IL-17 has been found by immunofluorescent staining of acutely rejecting human renal transplant biopsies when compared to healthy kidneys and pretransplant biopsies[20]. Additionally, elevated IL-17 mRNA and protein levels have been found in renal biopsy specimens and urinary sediment from patients found to have borderline rejection[21]. However, in several studies that measured serum IFNγ in kidney and liver transplant patients, there was no correlation between the presence of Th1 and rejection[17-19, 43]. Therefore it is likely that peripheral blood measurement of T-lymphocyte cytokines are not the best way to detect rejection and intragraft biopsies should be considered instead. However it is a possibility that the peripheral circulating T cells are those which are required for control of infection and malignancy. Perhaps they may be more important as a marker for patients at risk for post-transplant complications.

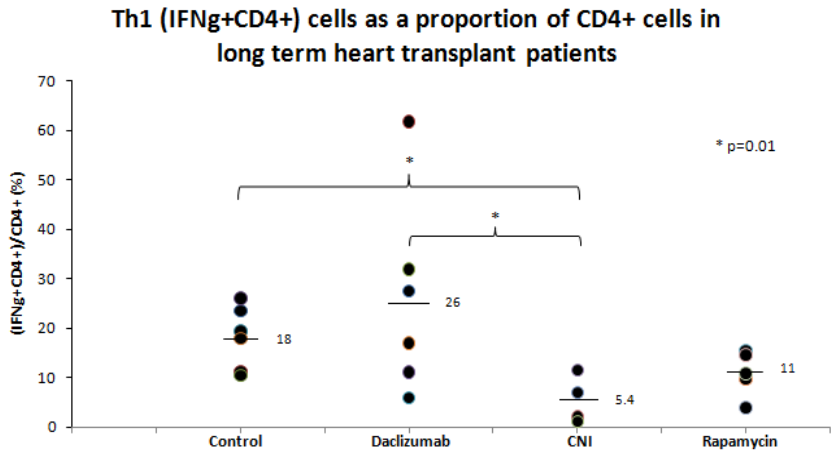
This study demonstrates that the impact of immunosuppressive therapeutic agents on key players in the immune system, in this case the CD4 T helper cells, is not equal. Although in our study we did not detect a correlation between the proportions of peripheral circulating T helper

cells and clinical implications such as rejection, infection and malignancy, it is possible that the assessment of different immune factors or biopsy samples could provide valuable insight on clinical implications. This could be of great use in the discovery of biomarkers that could be used for the prediction and prophylaxis of the complications associated with transplantation. Additionally, the discovery and subsequent targeting of relevant immune factors could prove to be effective immunosuppressive therapy as seen with anti-CD25 monoclonal antibodies. The impact of different immunosuppressive protocols on T cell subsets may become a useful tool in order to better tailor each protocol to individual patients who may be at risk for infections or malignancy without increasing their risk for rejection. Although the initial impetus was to use anti-CD25 mAb or rapamycin to protect the patients' renal function[32], the use of such strategies may also be suited for other considerations such as malignancy or infections. In liver transplant patients, the use of rapamycin is associated with a lower risk of skin cancers and lymphomas[38]. Although this effect is thought to be primarily due to its activity on mTOR, our findings that it has a reduced effect on Th1 and Th17 CD4 T cells may also be of interest.

Providing a better understanding of the mechanisms behind the complications associated with transplantation is essential for prophylaxis and identification of clinically relevant solutions in which there still exists significant shortcomings.

5. Figures

A.



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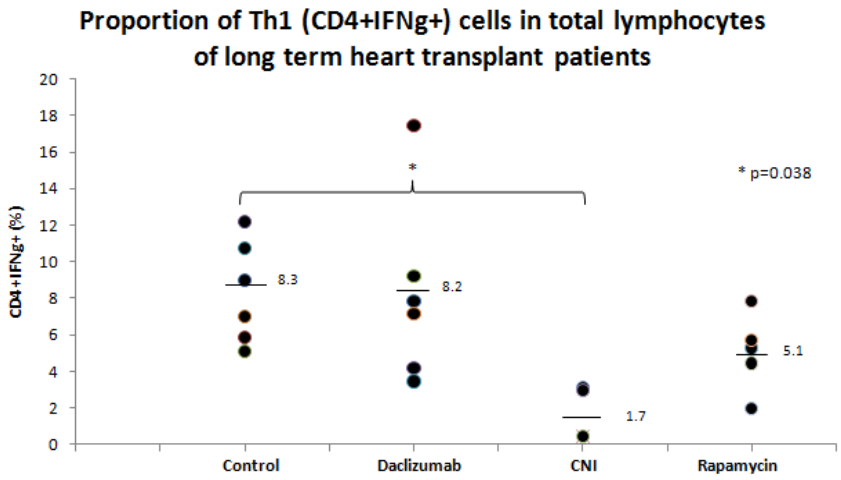
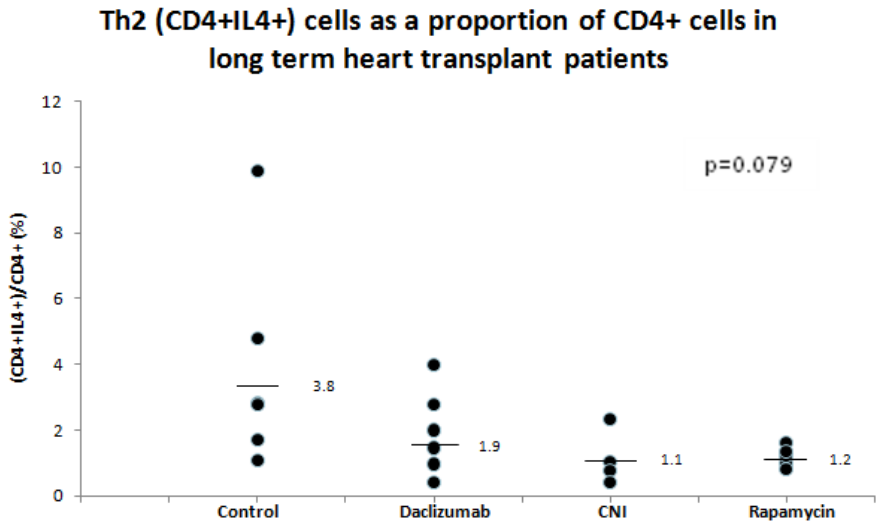


Figure 5.1: Th1 cells as a proportion of CD4 cells (A) and total lymphocytes (B) were measured in non-transplanted controls and long term heart transplant recipients on anti-CD25 mAb (daclizumab), calcineurin inhibitors (tacrolimus or cyclosporine) or rapamycin. Th1 cells were measured by flow cytometric analysis after 5 hours of stimulation and fluorescent staining with anti-human CD4 and IFNγ antibodies.

A.



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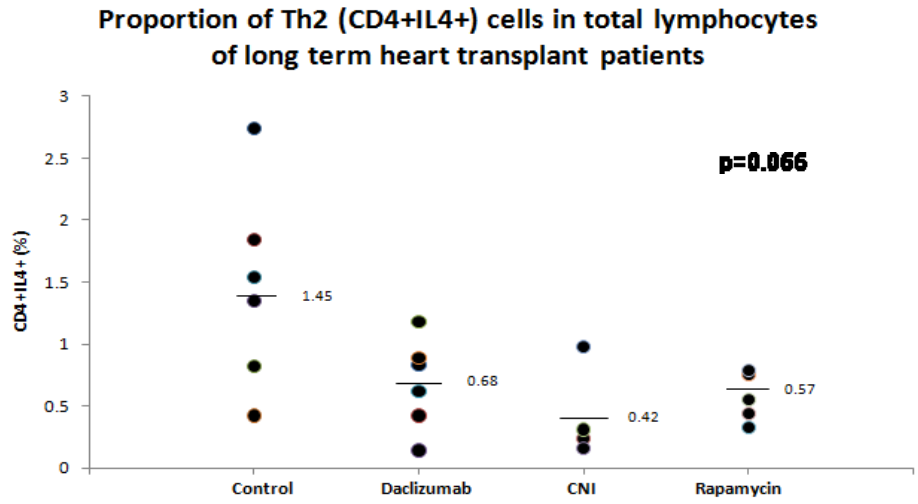
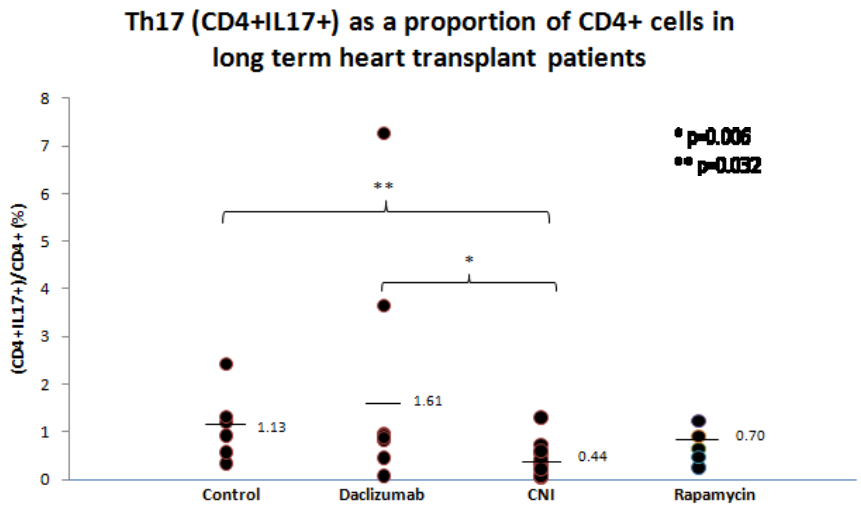


Figure 5.2: Th2 cells as a proportion of CD4 cells (A) and total lymphocytes (B) were measured in non-transplanted controls and long term heart transplant recipients. Th2 cells were measured by flow cytometric analysis after stimulation and staining with anti-human CD4 and IL-4 antibodies.

A.



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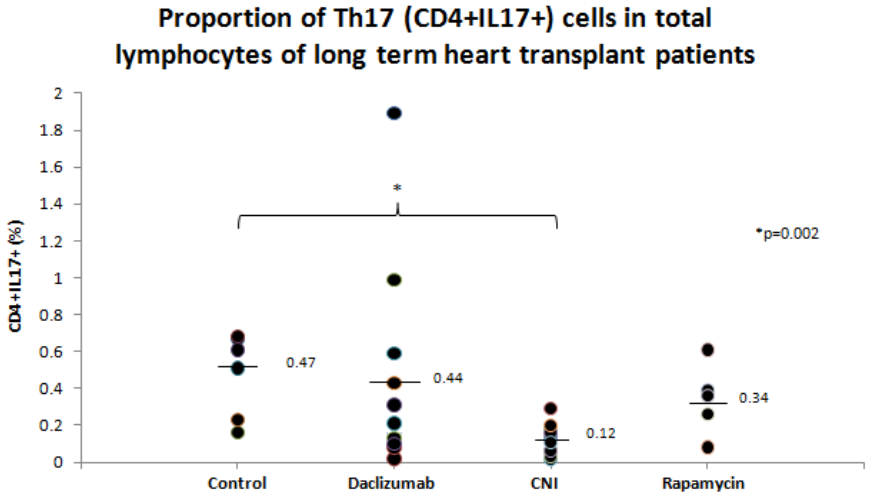
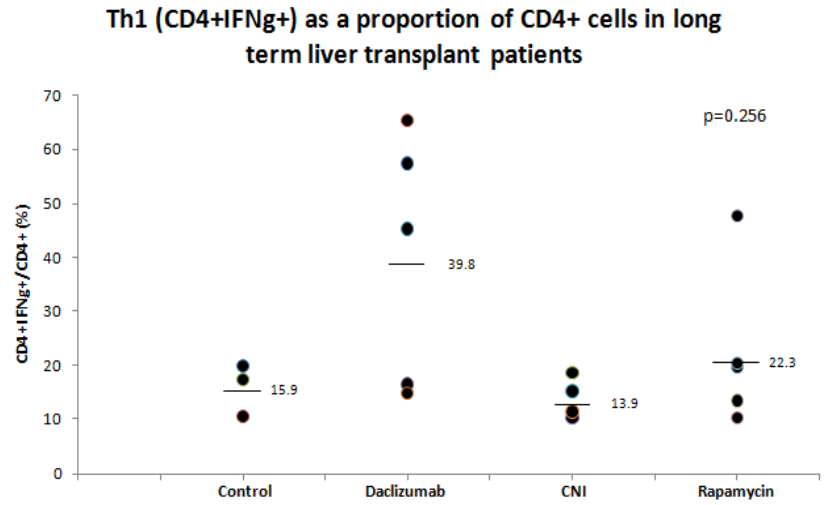


Figure 5.3: Th17 cells as a proportion of CD4 cells (A) and total lymphocytes (B) were measured in non-transplanted controls and long term heart transplant recipients. Th17 cells were measured by flow cytometric analysis after stimulation and staining with anti-human CD4 and IL-17 antibodies.

A.



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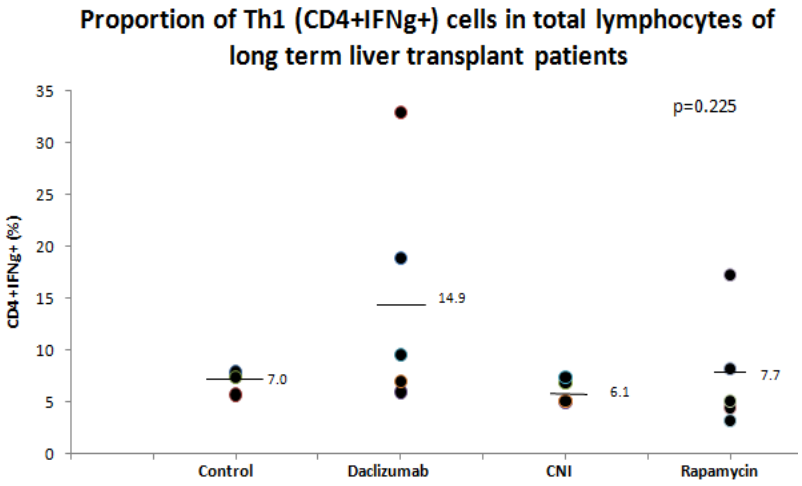
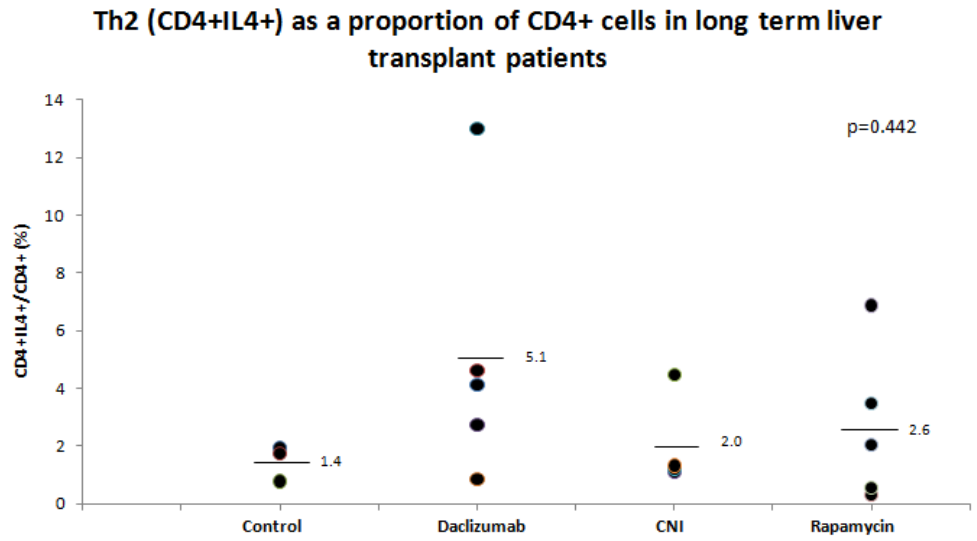


Figure 5.4: Th1 cells as a proportion of CD4 cells (A) and total lymphocytes (B) were measured in non-transplanted controls and long term liver transplant recipients on anti-CD25 mAb (daclizumab), calcineurin inhibitors (tacrolimus or cyclosporine) or rapamycin. Th1 cells were measured by flow cytometric analysis after 5 hours of stimulation and fluorescent staining with anti-human CD4 and IFNγ antibodies.

A.



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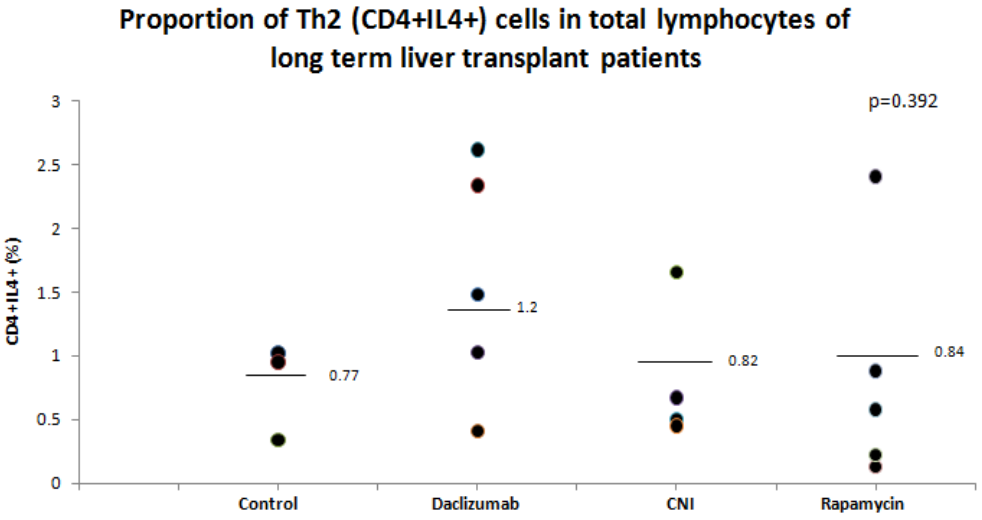
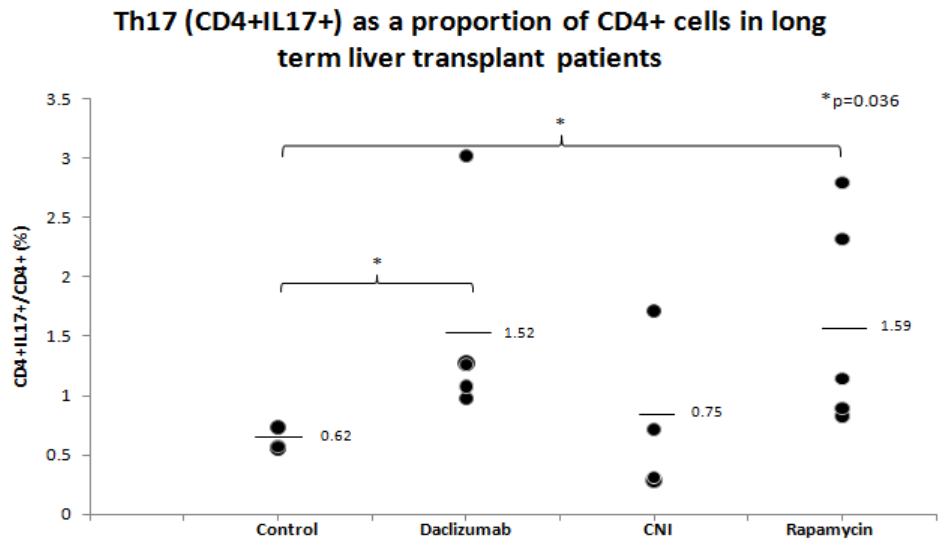


Figure 5.5: Th2 cells as a proportion of CD4 cells (A) and total lymphocytes (B) were measured in non-transplanted controls and long term liver transplant recipients. Th2 cells were measured by flow cytometric analysis after stimulation and staining with anti-human CD4 and IL-4 antibodies.

A.



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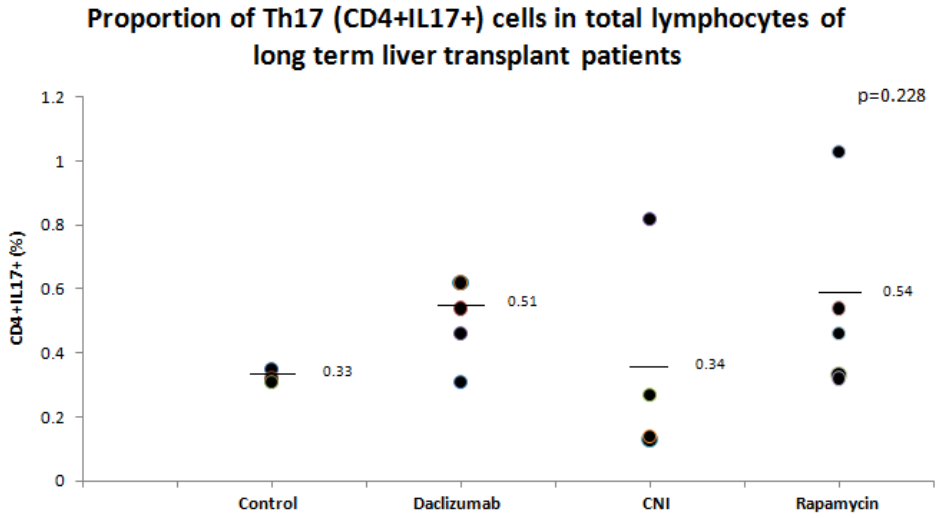

Figure 5.6: Th17 cells as a proportion of CD4 cells (A) and total lymphocytes (B) were measured in non-transplanted controls and long term liver transplant recipients. Th17 cells were measured by flow cytometric analysis after stimulation and staining with anti-human CD4 and IL-17 antibodies.

Figure 5.7: Observed events of infection, rejection and malignancy in heart transplant patients

Heart Tx patients	**Daclizumab**		**CNI**	**Rapamycin**
	Pre-switch (CNI)	Post-switch		
Infection				
-Viral	0	1	0	1
-Bacterial	1	2	0	1
-Fungal	0	1	0	0
Malignancy	0	0	0	0
Rejection	3	0	3	1

Figure 5.8: Observed events of infection, rejection and malignancy in liver transplant patients

Liver Tx patients	**Daclizumab**		**CNI**	**Rapamycin**
	Pre-switch (CNI)	Post-switch		
Infection				
-Viral	0	0	0	1
-Bacterial	4	2	2	3
-Fungal	2	0	1	1
Malignancy	1	0	2	0
Rejection	3	0	2	2

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